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A bispecific antibody strategy to target multiple type 2 cytokines in asthma

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Background: Asthma is a chronic inflammatory airway disease in which innate and adaptive immune cells act together to cause eosinophilic inflammation, goblet cell metaplasia (GCM) and bronchial hyperreactivity (BHR). In clinical trials employing biologicals against interleukin (IL)-4Rα or IL-5, only a subset of moderate-to-severe asthmatics responded favorably, suggesting that distinct pathophysiological mechanisms are at play in subgroups of patients, called endotypes. However, the effect of multiple cytokine blockade using bispecific antibodies (Ab) has not been tested.

Objective: To target simultaneously IL-4, IL-13 and IL-5 signaling pathways with a novel IL-4Rα/IL-5 bispecific Ab in a murine house dust mite (HDM) model of asthma.

Methods: Two monoclonal Abs neutralizing IL-4Rα and IL-5 were generated using a llama-based Ab platform. Their heavy (HC) and light chains (LC) where then co-transfected in mammalian cells, resulting in a heterogeneous Ab mixture from which the bispecific Ab was isolated using a dual anti-idiotypic purification process. C57BL/6J mice were finally sensitized and challenged to HDM extracts and treated during challenge with the Abs.

Results: We successfully generated and characterized the monospecific and bispecific Abs targeting IL-4Rα and IL-5. The monospecific Abs could suppress eosinophilia and/or IgE synthesis whereas only the IL-4Rα/IL-5 bispecific Ab and the combination of monospecific Abs additionally inhibited GCM and BHR.

Conclusion: Type 2 cytokines act synergistically to cause GCM and BHR in HDM-exposed mice.

Clinical applications: These preclinical results show the feasibility of generating bispecific Abs that target multiple cytokine signaling pathways as superior inhibitors of asthma features, including the difficult-to-treat GCM.

Clinical implications: This preclinical study demonstrates the feasibility of developing a bispecific Ab targeting multiple cytokine pathways as a potent inhibitor of asthma features, including the difficult-to-treat GCM, providing a new therapeutic approach to treat patients with moderate-to-severe asthma or with other chronic airway diseases in which mucus overproduction is often seen.

Capsule summary: Current asthma biologicals include IL-4Rα and IL-5 monospecific Abs. Here we show the feasibility of producing an anti-IL-4Rα/IL-5 bispecific Ab that neutralizes both targets and demonstrates superior effectiveness in a murine house dust mite model.

Key words: *asthma, bispecific antibody, bronchial hyperreactivity, goblet cell metaplasia, house dust mite, interleukin-4 receptor alpha, interleukin-5*

Abbreviations used

Ab: Antibody

BAL: Bronchoalveolar lavage

BHR: Bronchial hyperreactivity

ELISA: Enzyme-linked immunosorbent assay

Fab: Antigen-binding fragment

FACS: Fluorescence-activated cell sorting

Fc: Fragment crystallizable

GCM: Goblet cell metaplasia

HC: Heavy chain

HDM: House dust mite

ICS: Inhaled corticosteroid

Ig: Immunoglobulin

IL: Interleukin

LABA: Long-acting β_2-adrenergic agonist

LC: Light chain

MHC: Major histocompatibility complex

MLN: Mediastinal lymph node

Muc5AC: Mucin 5AC

PBS: Phosphate-buffered saline

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM: Standard error of the mean

SPDEF: Sam-pointed domain containing Ets transcription factor

SPR: Surface plasmon resonance

VHH: Camelid single-domain antibody fragment

INTRODUCTION

Asthma is a chronic inflammatory disease of the conducting airways affecting up to 300 million people worldwide that leads to symptoms of coughing, wheezing and chest tightness.[1-4] Airway obstruction runs a variable course, with symptom-free periods interrupted by periods of exacerbations, often caused by viral infection.[2] A typical feature is BHR, which is the tendency of the airways to constrict in response to stimuli such as cold air and exercise. Allergic sensitization to inhaled allergens such as HDMs (*Dermatophagoides pteronyssinus*), which concerns about 50% of asthmatic patients,[5] is often found in children with asthma and characterized by the presence of allergen-specific immunoglobulin (Ig) E in serum (so-called atopic predisposition). Only half of adults with asthma are atopic, but the disease otherwise presents similarly in the clinic.[6]

In bronchial biopsies or lung-resection samples, asthma is often characterized by accumulation of eosinophils, mast cells and $CD4^+$ T lymphocytes producing the type 2 cytokines IL-4 and/or IL-5 in the epithelium and lamina propria, even in individuals who have non-atopic asthma.[7-9] Animal models in which $CD4^+$ T cells or type 2 cytokines such as IL-4, IL-5 or IL-13 have been individually knocked out, have provided important evidence that the type 2 axis can drive eosinophilic airway inflammation and BHR.[10] However, asthma in humans is heterogeneous and a type 2-biased response disease seems to be detectable in only 50% of individuals with asthma, particularly those with early onset disease, an atopic predisposition and high blood eosinophil counts.[11] In some individuals with asthma, particularly those who respond poorly to steroids, airway infiltrates are composed primarily of neutrophils, which are probably recruited to the airways by IL-17-producing cells such as T-helper type 17 lymphocytes or γδT cells.[6, 12]

The finding of distinct clinical subsets of asthma that are driven by distinct pathophysiological mechanisms is also reflected in clinical trials employing biologicals aimed at blocking type 2 cytokines, wherein drugs targeting specifically IL-4Rα (e.g. dupilumab, targeting both IL-4 and IL-13 signaling pathways), IL-5 (e.g. mepolizumab or reslizumab) or IL-5Rα (benralizumab), are only effective for a subset of severe asthmatics that can be identified through the use of biomarkers.[13] Despite these differences in the clinical presentation, therapeutic responsiveness and underlying etiology of disease (called endotypes), there are signs of airway remodeling in almost all forms of asthma. These changes consist of an increase in the smooth-muscle mass surrounding the airway wall, a deposition of extracellular matrix components under the epithelial basement membrane that causes a thickened appearance, a breach in the integrity of the airway epithelium and an increase of mucus-producing goblet cells in the epithelium or submucosal glands.[14]

The increase in goblet cells and increased mucus production, is not due to the proliferation of pre-existing goblet cells but rather to the transdifferentiation of ciliated and secretory cells to goblet cells, under the influence of IL-4 and/or IL-13 and ligands of the epidermal growth factor receptor, and is therefore termed GCM.[14, 15] Goblet cells produce mucins, such as Mucin 5AC (Muc5AC), which control the viscoelasticity and hydration of the mucus covering the ciliary escalator.[14, 16] In asthma, the

sputum is often so dry that it can lead to mucus impaction and severe airway obstruction, and currently few therapeutic options exist to reduce GCM and improve mucus clearance.[16]

The types of patients that react best to currently (or soon to be) available biologicals all fall within the so-called type 2 asthma endotype, characterized by high blood and sputum eosinophilia and/or elevated allergen-specific serum IgE levels.[13] Many studies show that IgE synthesis, airway inflammation, remodelling and BHR are dissociated and may be mediated by different mechanisms under different conditions.[17] Yet, to date combination therapies targeting collaboratively inflammatory and potentiating pathways in asthma, such as an anti-IL-4Rα/IL-5 drug targeting simultaneously the IL-4, IL-13 and IL-5 pathways, have not been investigated.

Here, we generated and characterized novel conventional monospecific Abs directed against IL-4Rα and IL-5 using a llama-based Ab platform, which sources human-like V-regions from the immune system of "outbred" llamas (i.e. that each animal has a different genetic background, **Fig E1, A**). The outbred nature and the low degree of sequence homology between the human or mouse and the llama proteins explain why each llama is able to generate a unique but very diverse individual immune response, providing a wide array of Ab V-regions against the immunogens.[18] We then used the most neutralizing monospecific Abs to generate the anti-IL-4Rα/IL-5 bispecific Ab and purified it using a dual anti-idiotypic purification process (**Fig E2, A**). Using these Abs, we showed a novel synergistic effect of simultaneously targeting IL-4, IL-13 and IL-5 signaling pathways during challenge of a murine HDM model of asthma, with a potent capacity to reduce eosinophilic lung inflammation and allergen-specific IgE synthesis, prevent GCM and inhibit BHR in response to HDM challenge. This preclinical study demonstrates the feasibility of developing a bispecific Ab targeting multiple cytokine pathways as a potent inhibitor of asthma features, including the difficult-to-treat GCM, providing a new therapeutic approach to treat patients with moderate-to-severe asthma or with other chronic airway diseases in which mucus overproduction is often seen.

METHODS

Llama immunization and library construction

Two llamas, farmed outdoors according to the French animal welfare legislation, were immunized intramuscularly once per week over six weeks with recombinant mouse IL-4Rα-Fc and IL-5 (R&D Systems). They received 100 μg of IL-4Rα-Fc and 50 μg of IL-5, buffered in phosphate-buffered saline (PBS) and mixed with Incomplete Freund's Adjuvant (Sigma-Aldrich), the first two weeks, and 50 μg of IL-4Rα-Fc and 25 μg of IL-5 the remaining four weeks. Generation of Ab libraries was performed using the proprietary SIMPLE antibody™ platform at argenx as previously described (**Fig E1, A**).[19] Briefly, five days after the last immunization, peripheral blood lymphocytes were purified and used for extraction of total RNA. Total RNA was then converted into random primed cDNA using reverse transcriptase, and gene sequences encoding for VH-CH1 regions of llama IgG and VL-CL domains (kappa and lambda) were isolated and subcloned into a phagemid vector pCB3.

Phage selection for Fab generation

The *E. coli* strain TG1 (Netherlands Culture Collection of Bacteria) was transformed using recombinant phagemids to generate antigen-binding fragments (Fab)-expressing phage libraries (one lambda and one kappa library per immunized llama). Because the llamas were immunized with IL-4Rα fused to a fragment crystallizable (Fc) part, a counter-selection against an excess of Fc in solution was performed to get rid of phages expressing Fabs directed against Fc. The phages were adsorbed on immobilized recombinant biotinylated IL-4Rα-Fc or IL-5, and eluted using trypsin as previously described.[20] Three rounds of phage display selections were performed to enrich for phages expressing IL-4Rα or IL-5-specific Fabs. TG1 *E. coli* was finally infected with selected phages, and individual colonies were isolated. Secretion of Fabs was induced using isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich), and the Fab-containing periplasmic fractions of bacteria were collected and screened by surface plasmon resonance (SPR) using a Biacore 3000 apparatus (GE Healthcare).

Monospecific Ab production, purification and characterization

The cDNAs encoding the VH and VL (lambda or kappa) domains of the neutralizing Fabs fragments displaying the lowest off-rate were cloned into two separate mammalian expression vectors (U-Protein Express BV) which comprise the cDNAs encoding the CH1, hinge, CH2 and CH3 domains of a mouse IgG2a Ab, containing a mutation that abrogates Ab effector functions mediated by the Fc receptor (e.g. N297A)[21], or the CL (lambda or kappa), respectively. Production by transient transfection of HEK293 cells and endotoxin-free purification by protein A affinity chromatography was then performed to generate mouse IgG2a monoclonal Abs containing the N297A mutation. Their purity and homogeneity were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 2 μg of each sample, **Fig E1, B**), and their high affinity (8E-11 M for the anti-IL-4Rα monospecific Ab depicted in **Fig E1, C** and 2E-12M for the anti-IL-5 monospecific Ab depicted in **Fig E1, D**) and ability to neutralize their target (**Fig E1, E**) were determined by SPR. The neutralizing activity of both anti-IL-4Rα and anti-IL-5 monospecific Abs was finally confirmed in *in vitro* cellular assays, in which mouse

IL-4 and IL-5 induced the proliferation of HT-2 and TF-1 cells, respectively. Anti-IL-4Rα and anti-IL-5 monospecific Abs blocked the cellular proliferation induced by mouse IL-4 and IL-5 with an EC_{50} equals to 0.2 nM and 0.6 nM, respectively (**Fig E1, F**). Additionally, the ability of the anti-IL-4Rα monospecific Ab to block the translocation of MHC class II to the surface of B cells induced by mouse IL-4 was tested as previously described (**Fig E1, G**).[22]

Cell proliferation assays

Human TF-1 cells (erythroblasts, ATCC CRL-2003) and mouse HT-2 clone A5E cells (IL-2 dependent T lymphocytes, ATCC CRL-1841) were cultured at 37°C with 5% (v/v) CO_2 in growth medium containing RPMI 1640 (Sigma), 10 % (v/v) heat-inactivated fetal bovine serum (Sigma), 1X gentamycin (Sigma), and 2 ng/mL human granulocyte-macrophage colony-stimulating factor (R&D Systems) or human IL-2 (R&D Systems), respectively. These cytokines were replaced in the assay medium by mouse IL-5 for the TF-1 cells and mouse IL-4 for the HT-2 cells (0.25 ng/mL and 7.5 ng/mL, respectively, R&D Systems) and added to a serial titration of the Abs. Cells were washed and resuspended at a final cell density of 1.1×10^6 cells/mL for the TF-1 cells or 0.2×10^6 cells/mL for the HT-2 cells. Cells were then added to the assay medium before addition of the CellTiter 96 AQ_{ueous} One Solution Reagent (Promega). After 3h of incubation, the absorbance was measured (**Fig E1, F and G**).

Generation of anti-idiotypic VHH affinity purification columns

A phage display library encoding the variable domain of camelid single-domain Ab fragments (VHH) from naïve llama was generated as described.[23] VHHs specific of each idiotype formed by the properly paired VH and VL were selected by phage display as previously described.[24-26] One potent anti-idiotypic VHH for each monospecific Ab was produced, purified and coupled to 5 mL NHS-sepharose beads according to the manufacturer's protocol (GE Healthcare). The VHH-functionalized sepharose beads were packed into small Tricorn columns (GE Healthcare). Analysis of the specificity of the columns was studied by chromatography experiments (AKTA prime, GE Healthcare) as previously described.[26]

Bispecific Ab production and purification using a dual anti-idiotypic purification process

The bispecific Ab was produced in HEK293 cells by co-transfecting 1:1:1:1 of the HCs and LCs of the two parental monospecific Abs containing the knobs-into-holes mutations to force HC heterodimerization through a symmetric-to-asymmetric complementarity design (**Fig E2, A**).[27, 28] After protein A purification, the Ab mixture was injected onto the VHH anti-IL-4Rα monospecific Ab-functionalized column followed by the injection of the eluted fraction into the VHH anti-IL-5 monospecific Ab-functionalized column. Abs were eluted with 20 mM citrate buffer, 150 mM sodium chloride pH3.0 and subsequently neutralized with 1M potassium phosphate pH 8.0 (1/10 of the eluted volume). Finally, the bispecific Ab was concentrated, buffer exchanged to PBS (0.02% v/v)-Tween-80 and filter sterilized. The purity of the eluted bispecific Ab was tested by high-resolution mass spectrometry (**Fig E2, B**, 5 µg of each sample, RIC BVBA, Kortrijk, Belgium), whereas its binding ability was confirmed by SPR as previously described (**Fig E2, C and D)**.[26]

HDM mouse model

Experiments were approved by the ethical committee of the VIB-UGent Center for Inflammation Research. Female C57BL/6J wild-type mice were obtained from Jackson. All mice were used between 6-8 weeks of age. Experiments were carried out using age-matched groups. On day 0, mice were lightly anesthetized with isofluorane (2.5% v/v in air) and received 1 μg HDM (Greer Laboratories) intratracheally. On days 6-10, mice were lightly anesthetized with isofluorane (2.5% v/v in air) and challenged daily with 10 μg HDM intranasally. On days 6, 8 and 10, mice received intraperitoneally their Ab treatment 4h before challenge. As controls, mice were sensitized with HDM, challenged with PBS and treated with an irrelevant IgG2a Ab, or sensitized and challenged with HDM and treated with an irrelevant IgG2a Ab.

Collection and analysis of sera, BAL fluid, MLN cells and lungs

On day 14, mice were euthanized. Bronchoalveolar lavage (BAL) was performed using 3 × 1 mL of EDTA-containing PBS into the cannulated trachea, and the cellular composition was determined by fluorescence-activated cell sorting (FACS) as previously described.[29-31] Blood was obtained from the iliac vein, the serum was subsequently prepared and the quantities of HDM-specific IgE and IgG1 were determined as previously described.[31] Single cell suspensions (2×10^6 cells/mL) were obtained from mediastinal lymph nodes (MLN) by homogenizing the organ through a 100 μm cell sieve. Cells were restimulated *ex vivo* with 15 μg/mL HDM for 3 days in 96 round-bottom plates and supernatants were collected to determine the cytokine production by using the Read-SET-Go! Enzyme-linked immunosorbent assay sets (ELISA, eBioscience). Lungs were injected with PBS/OCT (1:1) solution, snap-frozen in liquid nitrogen and kept at -80°C until further processing for Muc5AC immunofluorescence staining as previously described.[29] Alternatively, lungs were snap-frozen in liquid nitrogen and kept at -80°C until further processing for real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) as previously described.[30] Briefly, RNA was obtained by using the TriPure Isolation Reagent (Roche) and isolated according to the manufacturer's instructions. RNA was reverse transcribed with a Transcriptor High Fidelity cDNA Synthesis Kit (Roche), and samples were analyzed by using SYBR green-based qRT-PCR with a LightCycler 480 system (Roche) against reference genes (*Rpl13a*, *Hprt* and *Sdha*). Otherwise, lung function was performed using an invasive measurement of dynamic resistance (Flexivent, Scireq) as previously described.[31]

Statistical analysis

Data are represented as means ± standard error of the means (SEM). Statistical significances between groups were calculated using the one-way ANOVA test with GraphPad Prism software v7.01 and Genstat software v19. Differences between groups were considered significant when $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$, *versus* the group of mice sensitized and challenged with HDM and treated with an irrelevant IgG2 Ab.

RESULTS

Combination of anti-IL-4Rα and anti-IL-5 monospecific Abs injected during challenge inhibited GCM and BHR

HDM is an important human allergen, which induces allergic airway inflammation in sensitized and challenged mice, in a model resembling human type 2-high allergic asthma.[30-32] Mice were subjected to the HDM sensitization and challenge protocol and received the monotherapy (high affinity and neutralizing anti-IL-4Rα or anti-IL-5 conventional monospecific Abs, newly generated using a llama-based platform as described in the Methods section and **Fig E1**), or a combination of both monotherapies during the entire sensitization and challenge period (**Fig E3, A**). As previously reported in this model, airway HDM exposure increased the total count of inflammatory cells (mainly lymphocytes and eosinophils) in BAL fluid of mice as observed in the mice treated with an irrelevant IgG2a Ab (**Fig E3, B**).[30-32] The numbers of BAL fluid lymphocytes and eosinophils were significantly decreased by the monotherapies as well as the combination of both monotherapies. Blocking IL-4Rα resulted in an improved efficacy as found for the IL-4Rα monotherapy and the combination of both monotherapies, which can be explained by the fact that IL-4 is needed to cause T-helper type 2 sensitization to inhaled antigen.[33]

To further study the effect of the combination therapy, the Ab treatments were given only during challenge as depicted in **Fig 1, A**. Based on previous titration experiments, a treatment dose of 150 µg per monospecific Ab was chosen (data not shown). Airway HDM exposures increased the total count of inflammatory cells (mainly lymphocytes and eosinophils) in BAL fluid compared to PBS-challenged mice (**Fig 1, B**; HDM-sensitized and -challenged mice treated with an irrelevant IgG2b Ab represented by the black bars, compared to the white bars representing HDM-sensitized/PBS-challenged mice treated with an irrelevant IgG2a Ab). The numbers of lymphocytes and eosinophils were significantly decreased after treatment with the monotherapies as well as the combination therapy. However, the lowest numbers of lymphocytes and eosinophils were seen in mice treated with the combination therapy, suggesting a possible synergistic effect of targeting IL-4Rα and IL-5.

Key aspects of airway inflammation are driven by the effector cytokines IL-5 and IL-13 produced by allergen-specific type 2 cells in the lungs and lymphoid organs.[34] The *in vitro* production of these cytokines was boosted in allergen-restimulated cultures of MLN cells isolated from IgG2a Ab-treated, HDM-sensitized and -challenged mice (**Fig 1, C**). This response was significantly decreased after *in vivo* treatment with the anti-IL-4Rα monospecific Ab and combination of both monotherapies whereas anti-IL-5 monospecific Ab had no significant effects. Similarly, the serum concentration of IL-4 dependent HDM-specific IgG1 and IgE was boosted by allergen challenge in IgG2a Ab treated mice and significantly reduced in mice treated with the anti-IL-4Rα monospecific Ab and combination of both monotherapies, but not (HDM-specific IgE) or less significantly (HDM-specific IgG1) affected by the anti-IL-5 monospecific Ab treatment (**Fig 1, D**).

GCM is driven by IL-13 and characterized by increased production of Muc5AC, a gel-forming mucin present in the airways of asthmatic mice and humans.[35] In irrelevant IgG2a Ab treated and HDM-sensitized mice, HDM challenge caused increased immunoreactivity for Muc5AC in the airway epithelium, compared with PBS challenge, as shown in **Fig 1, E**. The increased staining for Muc5AC was not affected by IL-4Rα or IL-5 monotherapies. Strikingly however, lungs from mice treated with the combination of monospecific Abs had strongly reduced staining intensity for Muc5AC compared to the other HDM-sensitized and -challenged groups. To confirm and quantify the effects of the combination of both monotherapies on GCM, the mRNA expression level of *Muc5ac*, as well as two other genes involved in GCM, *Agr2* and *Spdef*, were measured by qRT-PCR in lung tissues. Sam-pointed domain containing Ets transcription factor (SPDEF) regulates various genes that encode factors involved in producing mucins, their glycosylation, and intracellular packaging, including *Muc5ac* and *Agr2*.[36, 37] The mRNA expression levels of these three genes were induced by HDM challenge in mice compared to PBS challenge (**Fig 1, F**). The IL-4Rα monotherapy was not able to reduce the mRNA expression levels of these three genes whereas the IL-5 monotherapy reduced the expression of *Spdef*. Strikingly, the combination of both monotherapies strongly reduced the mRNA expression levels of the three genes (**Fig 1, F**), confirming the observation made at protein level for Muc5AC (**Fig 1, E**) and suggesting that the combination therapy acted downstream of phosphorylated STAT6 to inhibit IL-13/STAT6 signaling in airway epithelium.

BHR is the tendency of the airways to constrict in response to low amounts of bronchoconstrictor agents like the muscarinic receptor agonist methacholine. In irrelevant IgG2a Ab treated mice, HDM-challenged and -sensitized mice, methacholine caused increased airway resistance compared to PBS-challenged and HDM-sensitized mice (**Fig 1, G**). Treatment with the monotherapies did not reduce BHR, but strikingly, mice treated with the combination of both monotherapies were completely protected from developing BHR, showing an unexpected synergistic effect of the monotherapies when injected in combination.

Altogether, these results showed that the combination of IL-5 and IL-4Rα monospecific Abs given during challenge in HDM-exposed mice had unforeseen synergistic effects on key aspects of asthma, including GCM and BHR.

Anti-IL-4Rα/IL-5 bispecific Ab treatment injected during challenge inhibited GCM and BHR

During the past decade, dual targeting with bispecific Abs has emerged as an alternative to combination therapy. From a practical, technological, intellectual property and regulatory perspective, this makes the development less complex because manufacturing, preclinical and clinical testing, and administration is reduced to a single molecule.[38] Therefore, we generated an anti-IL-4Rα/IL-5 bispecific Ab using a dual anti-idiotypic purification process (**Fig E2, A**).[26]

We then tested the *in vivo* activity of the bispecific Ab and compared it with the monotherapies (anti-IL-4Rα or anti-IL-5 monospecific Abs) and the combination of both monotherapies. To conserve

equimolar inhibition of targets, and to eliminate differences in the total amount of Ab, we compared the effect of dosing 75 µg of each monospecific Ab combined with 75 µg of an irrelevant IgG2a Ab, a combination of 75 µg of each monospecific Abs and 150 µg of the bispecific Ab.

Mice were subjected as previously to the HDM sensitization and challenge protocol and treated with the Abs during challenge (**Fig 1, A**). Even with this lower treatment dose compared to the experiments showed in **Fig 1**, the numbers of eosinophils induced by HDM challenge in sensitized mice were significantly decreased after the injections of the monospecific Abs as well as the combination of both monotherapies (**Fig 2, A**). However, the lowest numbers of eosinophils, as well as lymphocytes, were seen in mice treated with the combination therapy, suggesting a possible synergistic effect of targeting IL-4Rα and IL-5 as previously observed (**Fig 1, B**). Notably, the bispecific Ab was as effective as the combination of both monotherapies in reducing airway eosinophilia and lymphocytosis induced by allergen challenge. The *in vitro* MLN type 2 cytokine (IL-5 and IL-13) production (**Fig 2, B**) as well as HDM-specific IgE and IgG1 (**Fig 2, C**) were also significantly decreased after treatment with either the combination of both monotherapies or the bispecific Ab.

To test and to compare the effects of the bispecific Ab on GCM, the mRNA expression levels of *Muc5ac*, *Spdef* and *Agr2* were measured in lung tissue. Interestingly, their expressions were reduced by either the combination of both monotherapies or the bispecific Ab, but not by the monotherapies (**Fig 2, D**). Only expression of *Spdef* was reduced by the IL-5 monotherapy whereas the IL-4Rα monotherapy was not able to reduce mRNA expression level of these three genes as previously shown in **Fig 1, F**. More remarkably, treatment with either the combination of both monotherapies or the bispecific Ab protected lungs equally well from HDM induced BHR, as assessed by methacholine induced bronchoconstriction (**Fig 2, E**).

Altogether these results showed that a bispecific Ab targeting simultaneously IL-4, IL-13 and IL-5 signaling pathways is effective in reducing all salient asthma features when given during challenge in a HDM driven model of asthma.

DISCUSSION

Since the early 1990's, it has become clear that asthma is frequently a type 2 mediated disorder, often occurring in patients with atopic predisposition, and characterized by the presence of airway and blood eosinophils, GCM, airway remodeling and BHR that leads to frequent exacerbations.[2, 7, 8, 39] In many patients, the disease can be controlled by a combination of nonspecific drugs, an inhaled corticosteroid (ICS) and a short- or long-acting β_2-adrenergic agonist (LABA). Nevertheless, in 5 to 10% of asthmatics, called severe asthmatics, the disease runs a severe course, requiring a need for new and more effective drugs to prevent the occurrence of potentially life-threatening episodes.

Despite the initial setbacks targeting type 2 cytokines in asthma, the therapeutic potential and proven benefit of targeting individual type 2 mediators (such as IgE, IL-5, IL-5Rα and IL-4Rα) has now been established in endotypes of asthmatics who match the therapeutic mechanism. Indeed, the therapeutic monospecific Ab targeting IL-4Rα (dupilumab) was shown to reduce asthma exacerbation frequency and IgE levels, and improved lung function after ICS and LABA withdrawal in patients with persistent moderate-to-severe asthma and elevated blood or sputum eosinophil counts. In these patients, dupilumab treatment had no effect on the elevated eosinophil counts however, and in four patients eosinophil levels even went up after a 12-week therapy period.[40] Abs targeting IL-5 (e.g. mepolizumab or reslizumab) or IL-5Rα (benralizumab) have also been extensively tested and can now be used as add-on therapy to ICS and LABA in patients with persistent moderate-to-severe asthma and high blood eosinophil counts, to reduce exacerbation frequency and ICS use.[41-47]

In the studies targeting IL-4Rα, IL-5 or IL-5Rα, all patients were selected as having a type 2 signature, characterized by blood and/or tissue eosinophilia and often accompanied by high serum IgE levels. Although these therapies have shown benefit in the entire study population, it is possible that some patients in which one therapy is not so effective or even in those where it is effective, there might be greater benefit from blocking additional type 2 cytokines. This is certainly reflected in the dupilumab clinical trial studies, where treatment did not reduce blood eosinophilia.[40] It is indeed known that the cytokine pathways can be very redundant.[48] Combining two monoclonal Abs targeting IL-5 and IL-4Rα, i.e. targeting simultaneously IL-4, IL-13 and IL-5 signaling pathways, has not been performed in patients and could be very costly and impractical to set up, given that the established Abs have their own dosing interval. Therefore, we set out to study the feasibility of developing one single therapeutic molecule, a bispecific Ab, that targets simultaneously IL-4Rα and IL-5.

We exploited a llama-based Ab platform to generate neutralizing IL-4Rα and IL-5 monospecific conventional Abs. After confirmation of their purity and functionality, we showed a synergistic effect of these two monospecific Abs injected in combination during challenge of HDM-exposed mice. Whereas the individual Abs each targeted certain aspects of the disease, only the combination of both monotherapies synergistically inhibited GCM and BHR when given during the allergen challenge phase. This finding was unexpected since none of the monotherapies reduced BHR (**Fig 1**). Prompted by these data, we sought to generate one single therapeutic molecule, an anti-IL-4Rα/IL-5 bispecific Ab. The

bispecific Ab, containing the HCs and the LCs of the parental monoclonal Abs, was isolated using a dual anti-idiotypic purification process.[26] Notably, the bispecific Ab maintained the binding capacities of its parental molecules and was therefore tested in the same murine HDM model of asthma. Interestingly, the bispecific Ab reduced eosinophilic lung inflammation and prevented GCM in response to HDM challenge, confirming the results obtained with the combination of both monospecific Abs and showing the feasibility to generate a bispecific Ab with enhanced potential for asthma treatment in this preclinical model. Indeed, the combination of both monotherapies as well as the bispecific Ab reduced the mRNA expression levels of *Muc5ac* and *Agr2* (coding for goblet cell proteins that are associated with increased mucus production), and of *Spdef* (coding for a transcription factor that promotes GCM) (**Fig 2, D**).[37] More remarkably, treatment with either the combination of both monotherapies or the bispecific Ab protected lungs equally well from HDM induced BHR, as assessed by methacholine induced bronchoconstriction (**Fig 2, E**).

These data obtained in a relevant asthma model employing HDM allergen for sensitization and challenge are different from previous studies performed in the ovalbumin-driven mouse asthma model. It was indeed shown in ovalbumin-driven models that individual blockade of IL-4Rα or even only IL-13 was sufficient to block GCM and BHR.[35, 49] We speculate that these different outcomes are caused by the fact that in the ovalbumin-driven model of asthma, the allergen challenge strictly depends on the adaptive T_H2 immune response triggered by harmless ovalbumin proteins, whereas in the HDM model, the allergen challenge phase results from an innate and adaptive immune response to HDM that contains many proteases and danger associated pathogen-associated molecular patterns that can elicit responses from epithelial cells and many innate immune cells like ILC2s, basophils, mast cells and eosinophils simultaneously.[2] To block GCM and BHR in this setting, we speculate that it is important to suppress several cytokines simultaneously. Even in the OVA model, it was previously demonstrated that BHR and tissue eosinophilia were intact in ovalbumin-sensitized and -challenged *Il-4ra*$^{-/-}$ mice, yet fully abolished when these mice were additionally rendered genetically deficient in *Il5*.[50] Mechanistically, the potential of T cells to produce IL-13 is strongly enhanced by the presence of eosinophils that depend on IL-5.[51, 52] It was also recently shown in mice that eosinophils act in concert with IL-13 from T cells to induce GCM, through release of eosinophil peroxidase.[53] It is also possible that eosinophils produce cytokines like TGF-β or other granule contained mediators that could contribute to GCM. Although these studies showed cooperation between IL-5/eosinophils and the IL-4/IL-13 axis, they were performed in constitutive gene deficient mice, suggesting that the effects might have been caused by alterations of the sensitization process. We show here however, that using therapeutic Abs in already sensitized mice, we can induce a synergism that should lead to better therapy.

In summary, we identified a novel neutralizing IL-4Rα/IL-5 bispecific Ab, which by means of a synergistic mechanism effectively reduced eosinophilic lung inflammation, prevented GCM and inhibited BHR in response to HDM challenge. The results of our preclinical study may lead to clinical

trials with a similar human IL-4Rα/IL-5 bispecific Ab approach in patients with moderate-to-severe asthma or with other chronic airway disorders in which mucus overproduction is often seen.

Acknowledgments

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FIG 1. Combination of anti-IL-4Rα and anti-IL-5 monospecific Abs injected during challenge in a murine HDM model of asthma inhibited GCM and BHR. A, Experimental setup of HDM-exposed C57BL/6J mice treated with Abs during challenge. **B**, Differential cell counts in BAL fluid of HDM-exposed mice that received the anti-IL-4Rα monospecific Ab, anti-IL-5 monospecific Ab, combination of monospecific Abs or an irrelevant IgG2a Ab analysed by FACS. **C**, Cytokine (IL-5 and IL-13) production by MLN cells restimulated with HDMs for three days *ex vivo* determined by ELISA. **D**, Serum levels of HDM-specific IgE and IgG1 determined by ELISA. **E**, Muc5AC confocal staining in mouse lungs after Ab treatments. **F**, Lung mRNA expression levels of *Muc5ac*, *Spdef* and *Agr2* determined by qRT-PCR. **G**, BHR measured after exposure to increasing doses of methacholine using Flexivent (SCIREQ). Data are representative of three independent experiments, with at least n = 6 mice per group and are means ± SEMs. ns: not significant, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$ *versus* HDM-sensitized and -challenged mice treated with an irrelevant IgG2a Ab.

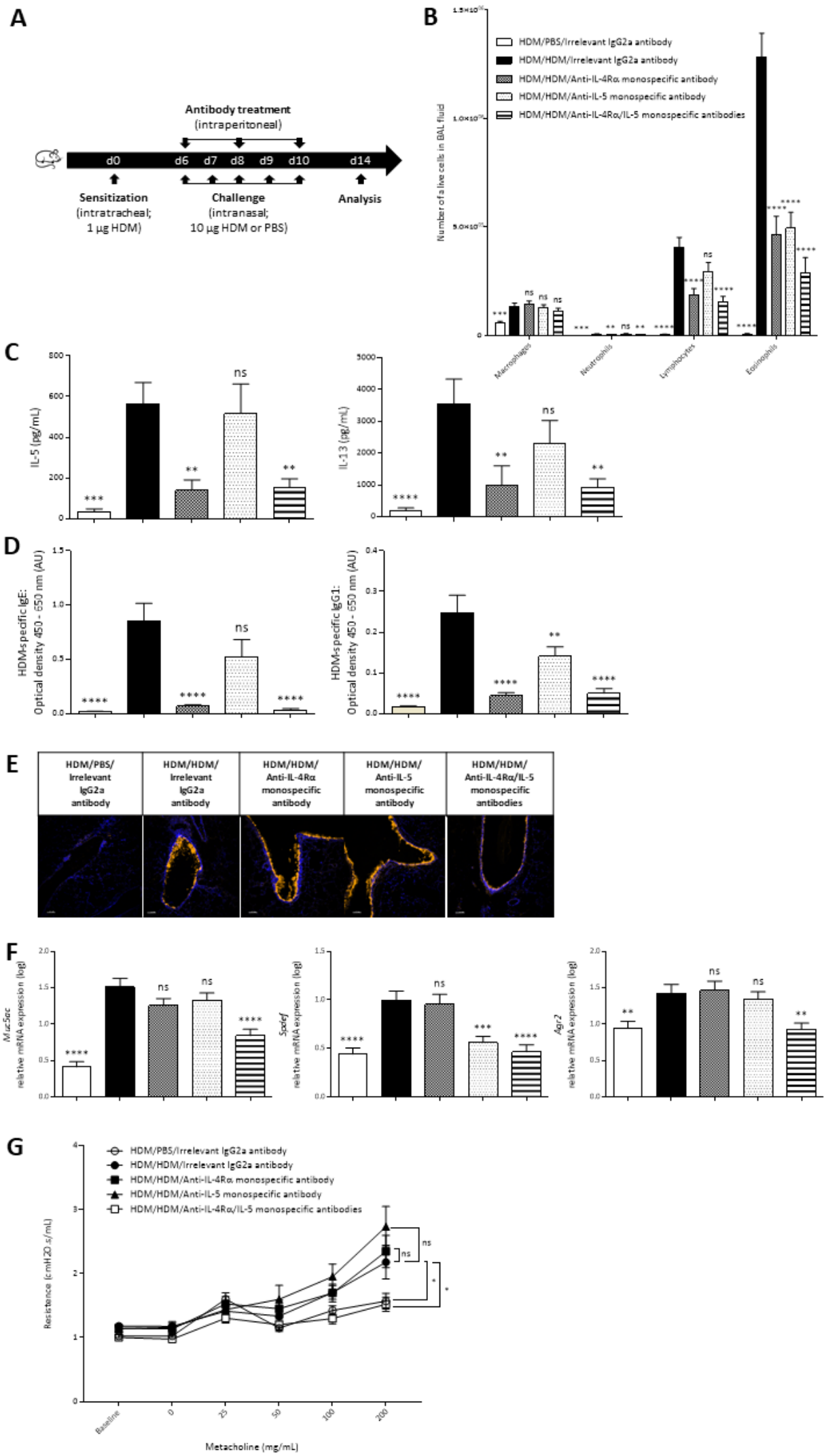
FIG 2. Anti-IL-4Rα/IL-5 bispecific Ab injected during challenge in a murine HDM model of asthma inhibited GCM and BHR. A, Differential cell counts in BAL fluid of HDM-exposed mice treated with the anti-IL-4Rα monospecific Ab, anti-IL-5 monospecific Ab, combination of monospecific Abs, anti-IL-4Rα/IL-5 bispecific Ab or an irrelevant IgG2a Ab analysed by FACS. **B**, Cytokine (IL-5 and IL-13) production by MLN cells restimulated with HDMs for three days *ex vivo* determined by ELISA. **C**, Serum levels of HDM-specific IgE and IgG1 determined by ELISA. **D**, Lung mRNA expression levels of *Muc5ac*, *Spdef* and *Agr2* determined by qRT-PCR. **E**, BHR measured after exposure to increasing doses of methacholine using Flexivent (SCIREQ). Data are representative of two independent experiments, with n = 6 mice per group and are means ± SEMs. ns: not significant, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$ *versus* HDM-sensitized and -challenged mice treated with an irrelevant IgG2a Ab.

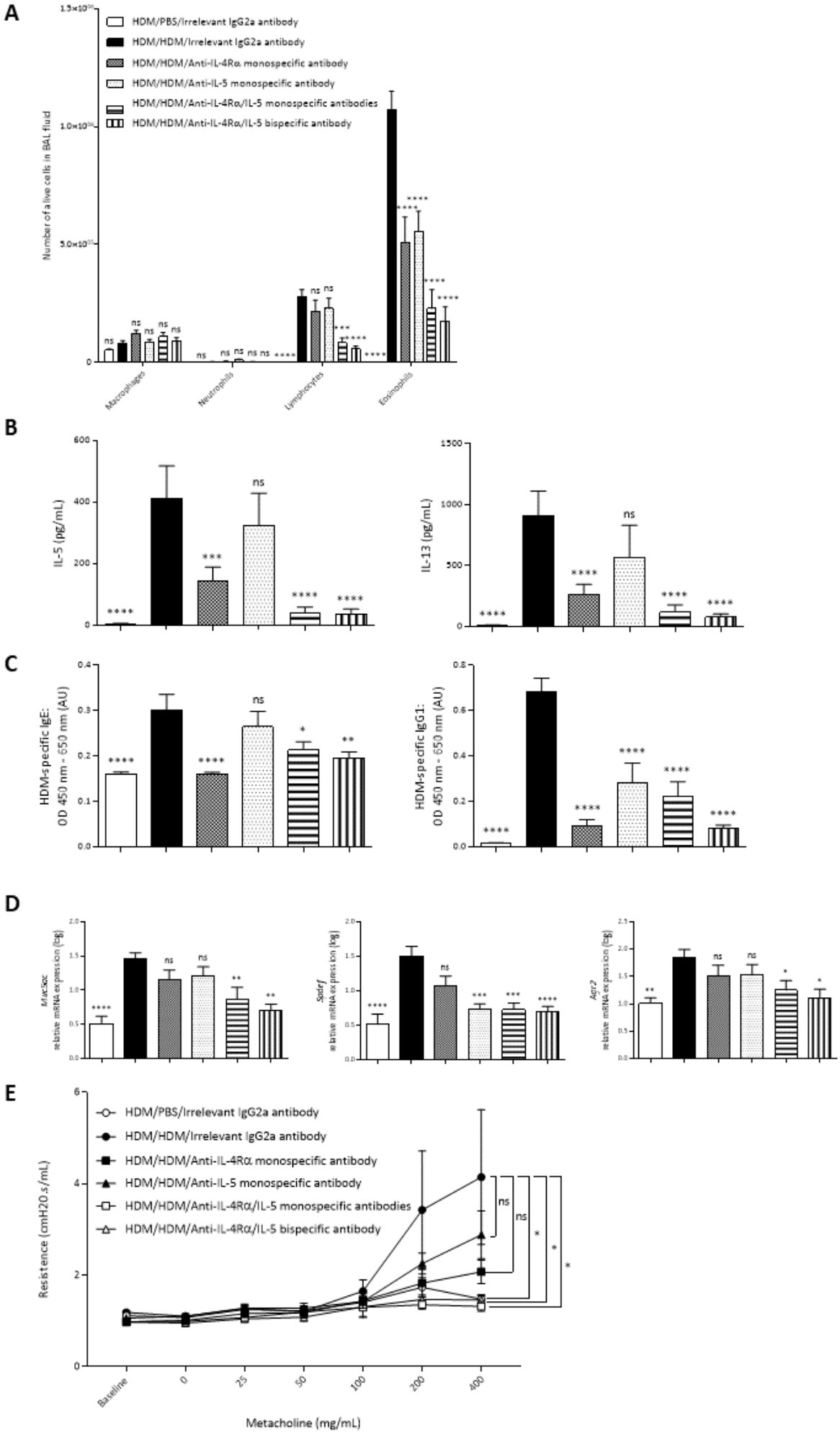
FIG E1. Anti-IL-4Rα and anti-IL-5 monospecific Abs generated using a llama-based Ab platform and characterized *in vitro*. A, Two llamas (*Lama glama*) were immunized with six weekly injections of mouse IL-4Rα-Fc and mouse IL-5. After immunization, total RNA was extracted from peripheral blood lymphocytes, and the cDNAs encoding the VL-CL and VH-CH1 domains were PCR-amplified. Amplicons were cloned into the phagemid vector pCB3 leading to the expression of Fab fragments fused to phage pIII coat protein. Recombinant phages were selected on immobilized target proteins during three rounds of panning resulting in the enrichment of phages displaying IL-5 or IL-4Rα-specific Fabs. The cDNAs encoding the VH and VL domains of selected Fab fragments were engineered into two separate mammalian expression vectors containing the cDNAs encoding the CH1, hinge, CH2 and CH3 domains of a mouse IgG2a-N297A Ab, or the CL, respectively. **B**, Confirmation of the purity and homogeneity of the anti-IL-4Rα and anti-IL-5 Abs analysed by SDS-PAGE under non-reducing and reducing conditions. **C**, Representative SPR sensorgram of the interactions between the anti-IL-4Rα monospecific Ab (or irrelevant IgG2a Ab) at varying concentrations (0-20 µg/mL) and the immobilized target IL-4Rα-Fc. **D**, Representative SPR sensorgram of the interactions between the anti-IL-5 monospecific Ab (or irrelevant IgG2a Ab) at varying concentrations (0-20 µg/mL) and the immobilized target IL-5. **E**, Representative SPR sensorgrams of the interactions between a mixture composed of a monospecific Ab (IL-4Rα, IL-5 or irrelevant IgG2a Ab) and its target (IL-4Rα or IL-5), and the immobilized proteins (IL-4, IL-13Rα or IL-5Rα). **F**, Anti-IL-4Rα (square) and anti-IL-5 (triangle) monospecific Abs potently inhibited mouse IL-4- and IL-5-induced HT-2 and TF-1 cell proliferation, respectively. Results are expressed as mean of triplicates ± SEM of two independent experiments. **G**, Anti-IL-4Rα monospecific Ab (blue) potently inhibited IL-4 induced MHC class II antigen expression on B cells as analyzed by FACS. PlacZ: *E. coli* lac Z promoter; RBS: ribosome binding site; S: signal peptide; H: poly-histidine; T: MYC-tag; AMBER: amber stop codon; gIII: phage gene encoding the coat pIII protein.

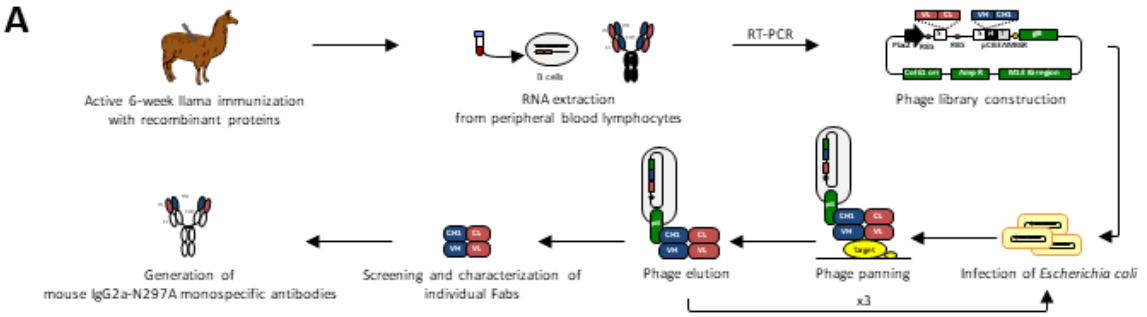
FIG E2. Anti-IL-4Rα/IL-5 bispecific Ab purified using a dual anti-idiotypic purification process maintained the binding capacities of the parental molecules. A, Schematic representation of the dual anti-idiotypic purification process to isolate a desired and properly paired bispecific Ab (framed) containing the knobs-into-holes mutations, which create a symmetric-to-asymmetric complementarity design. (**a**) From a mixture of four possible combinations formed by alternative LCs pairings, an anti-

idiotypic VHH recognizing only the correct HC/LC pairing of the anti-IL-4Rα monospecific Ab is used to extract the two molecules containing this pairing (**b**). Then, a second anti-idiotypic column (VHH anti-IL-5 monospecific Ab) is used to only collect the bispecific Ab containing the properly paired HC/LC of the anti-IL-5 monospecific Ab. (**c**) This leads to the isolation of the desired anti-IL-4Rα/IL-5 bispecific Ab with correct HC/LC pairings. **B**, Confirmation of the purity and homogeneity of the bispecific Ab analysed by high-resolution mass spectrometry. The bispecific Ab had a molecular mass (143.9 kDa) intermediate to the parental anti-IL-4Rα (144.6 kDa) and anti-IL-5 (143.5 kDa) monospecific Abs. **C**, SPR signals measured after subsequent injection of the anti-IL-4Rα monospecific Ab, anti-IL-5 monospecific Ab or anti-IL-4Rα/IL-5 bispecific Ab, and IL-4Rα-Fc or IL-5, on coated IL-4Rα-Fc. **D**, SPR signals measured after subsequent injection of the anti-IL-4Rα monospecific Ab, anti-IL-5 monospecific Ab or anti-IL-4Rα/IL-5 bispecific Ab, and IL-4Rα-Fc or IL-5, on coated IL-5.

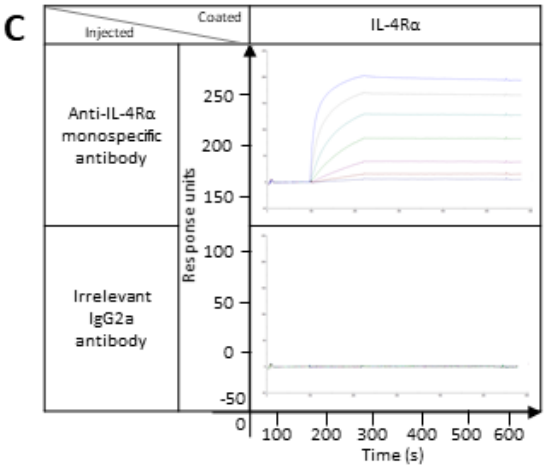
FIG E3. Combination of anti-IL-4Rα and anti-IL-5 monospecific Abs injected during sensitization and challenge in a murine HDM model of asthma reduced lymphocytosis and eosinophilia. A, Experimental setup of HDM-exposed C57BL/6J mice with Ab treatment during sensitization and challenge. **B**, Differential cell counts in BAL fluid of mice that received the anti-IL-4Rα monospecific Ab, anti-IL-5 monospecific Ab, combination of monospecific Abs or an irrelevant IgG2a Ab analysed by FACS. Data are representative of two independent experiments, with n = 6 mice per group and are means ± SEMs. ns: not significant, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$ *versus* HDM-sensitized and -challenged mice treated with an irrelevant IgG2a Ab.

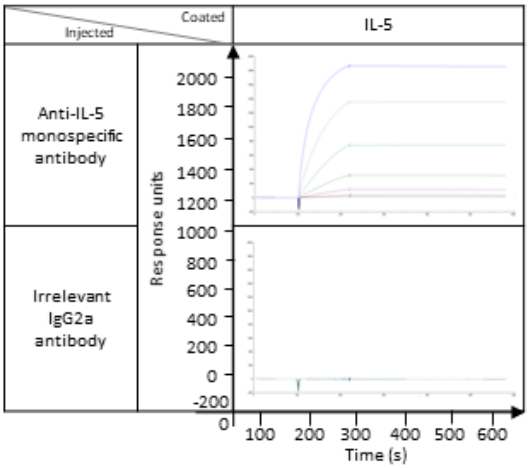

A
Antibody treatment
(intraperitoneal)
d0
d6
d7
d8
d9
d10
d14
Sensitization
(intratracheal;
1 µg HDM)
Challenge
(intranasal;
10 µg HDM or PBS)
Analysis
B
HDM/PBS/Irrelevant IgG2a antibody
HDM/HDM/Irrelevant IgG2a antibody
HDM/HDM/Anti-IL-4Rα monospecific antibody
HDM/HDM/Anti-IL-5 monospecific antibody
HDM/HDM/Anti-IL-4Rα/IL-5 monospecific antibodies
Number of alive cells in BAL fluid
Macrophages
Neutrophils
Lymphocytes
Eosinophils
C
IL-5 (pg/mL)
IL-13 (pg/mL)
D
HDM-specific IgE:
Optical density 450 - 650 nm (AU)
HDM-specific IgG1:
Optical density 450 - 650 nm (AU)
E
HDM/PBS/
Irrelevant
IgG2a
antibody
HDM/HDM/
Irrelevant
IgG2a
antibody
HDM/HDM/
Anti-IL-4Rα
monospecific
antibody
HDM/HDM/
Anti-IL-5
monospecific
antibody
HDM/HDM/
Anti-IL-4Rα/IL-5
monospecific
antibodies
F
Muc5ac
relative mRNA expression (log)
Spdef
relative mRNA expression (log)
Agr2
relative mRNA expression (log)
G
HDM/PBS/Irrelevant IgG2a antibody
HDM/HDM/Irrelevant IgG2a antibody
HDM/HDM/Anti-IL-4Rα monospecific antibody
HDM/HDM/Anti-IL-5 monospecific antibody
HDM/HDM/Anti-IL-4Rα/IL-5 monospecific antibodies
Resistance (cmH2O.s/mL)
Baseline
0
25
50
100
200
Metacholine (mg/mL)

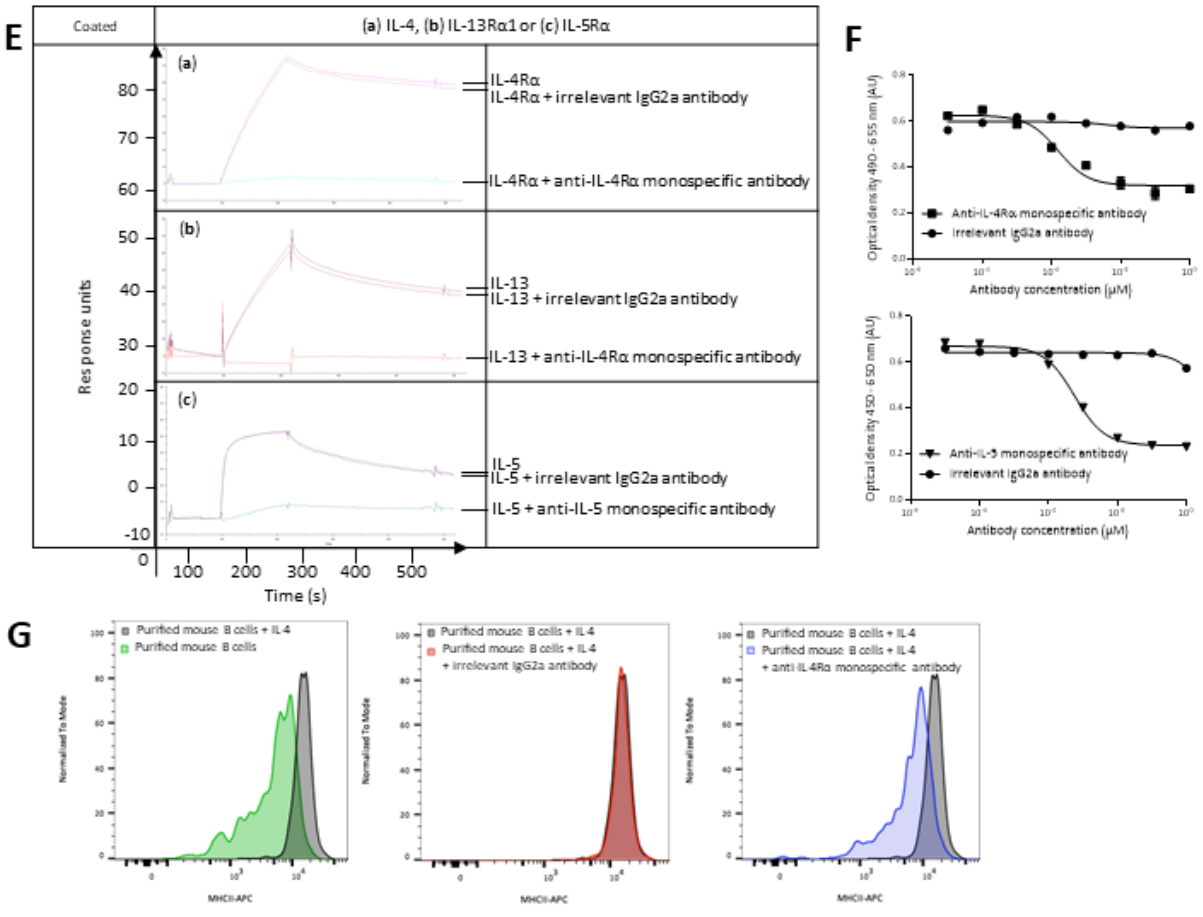

A
HDM/PBS/Irrelevant IgG2a antibody
HDM/HDM/Irrelevant IgG2a antibody
HDM/HDM/Anti-IL-4Rα monospecific antibody
HDM/HDM/Anti-IL-5 monospecific antibody
HDM/HDM/Anti-IL-4Rα/IL-5 monospecific antibodies
HDM/HDM/Anti-IL-4Rα/IL-5 bispecific antibody
Number of alive cells in BAL fluid
Macrophages
Neutrophils
Lymphocytes
Eosinophils
B
IL-5 (pg/mL)
IL-13 (pg/mL)
C
HDM-specific IgE: OD 450 nm - 650 nm (AU)
HDM-specific IgG1: OD 450 nm - 650 nm (AU)
D
Muc5ac relative mRNA expression (log)
Sostdc1 relative mRNA expression (log)
Agr2 relative mRNA expression (log)
E
HDM/PBS/Irrelevant IgG2a antibody
HDM/HDM/Irrelevant IgG2a antibody
HDM/HDM/Anti-IL-4Rα monospecific antibody
HDM/HDM/Anti-IL-5 monospecific antibody
HDM/HDM/Anti-IL-4Rα/IL-5 monospecific antibodies
HDM/HDM/Anti-IL-4Rα/IL-5 bispecific antibody
Resistence (cmH2O.s/mL)
Baseline
0
25
50
100
200
400
Metacholine (mg/mL)

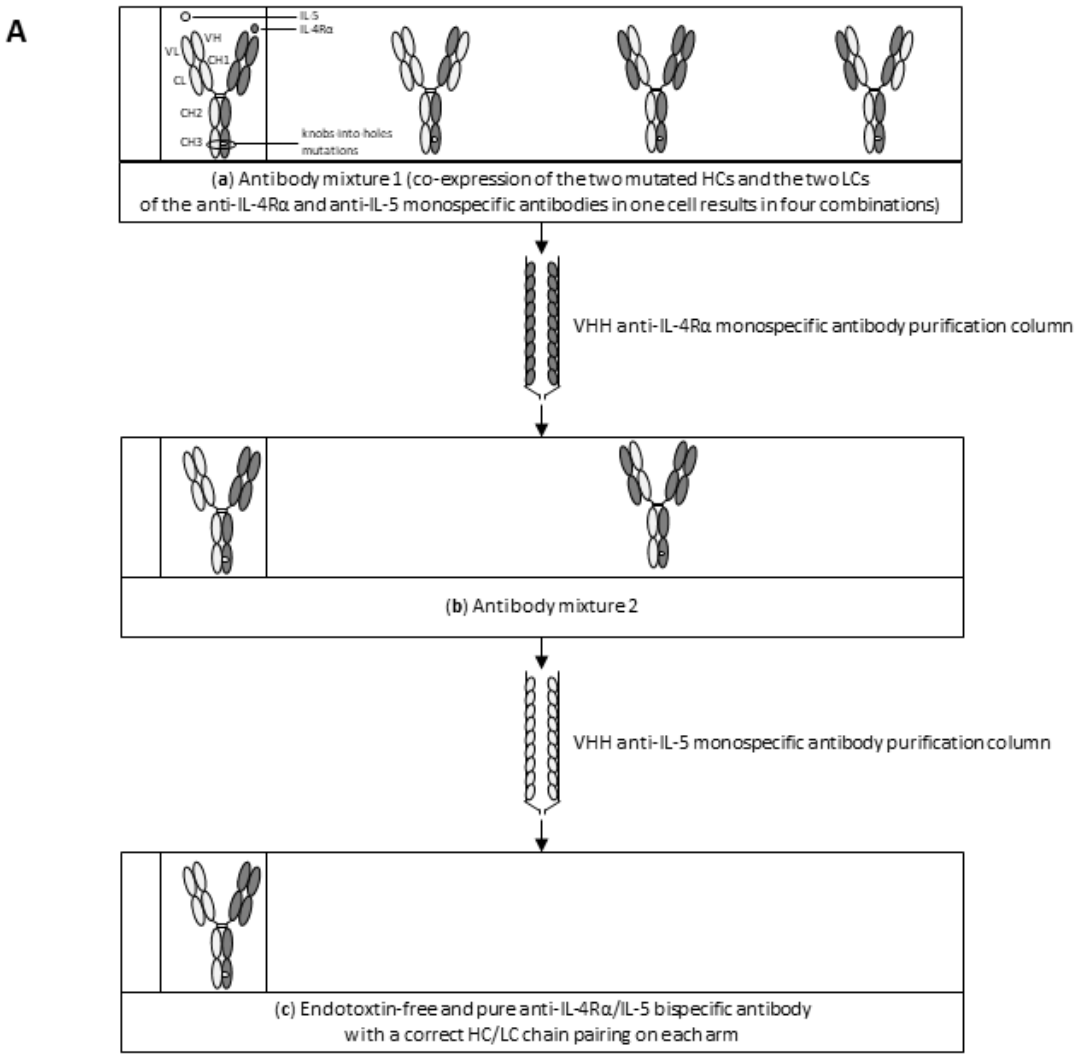

A
Active 6-week llama immunization with recombinant proteins
B cells
RNA extraction from peripheral blood lymphocytes
RT-PCR
Phage library construction
Infection of Escherichia coli
Phage panning
Phage elution
x3
Screening and characterization of individual Fabs
Generation of mouse IgG2a-N297A monospecific antibodies

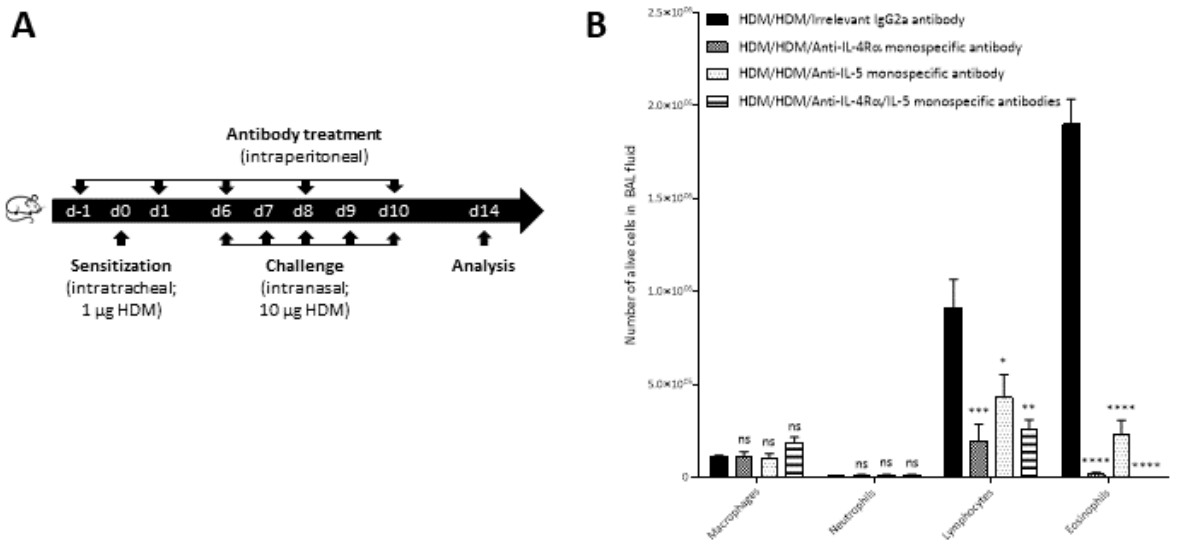

B
Non-reducing conditions
Reducing conditions
Anti-IL-4Rα monospecific antibody
Anti-IL-5 monospecific antibody
Marker
Anti-IL-4Rα monospecific antibody
Anti-IL-5 monospecific antibody
Molecular weight (kDa)
~170
~130
~100
~70
~55
~40
~35
~25
~15
~10


C
Injected
Coated
IL-4Rα
Anti-IL-4Rα monospecific antibody
Irrelevant IgG2a antibody
Response units
250
200
150
100
50
0
-50
0
100
200
300
400
500
600
Time (s)


D
Injected
Coated
IL-5
Anti-IL-5 monospecific antibody
Irrelevant IgG2a antibody
Response units
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
0
100
200
300
400
500
600
Time (s)


E
Coated
(a) IL-4, (b) IL-13Rα1 or (c) IL-5Rα
(a)
(b)
(c)
IL-4Rα
IL-4Rα + irrelevant IgG2a antibody
IL-4Rα + anti-IL-4Rα monospecific antibody
IL-13
IL-13 + irrelevant IgG2a antibody
IL-13 + anti-IL-4Rα monospecific antibody
IL-5
IL-5 + irrelevant IgG2a antibody
IL-5 + anti-IL-5 monospecific antibody
Response units
Time (s)
F
Optical density 490 - 655 nm (AU)
Anti-IL-4Rα monospecific antibody
Irrelevant IgG2a antibody
Antibody concentration (μM)
Optical density 450 - 650 nm (AU)
Anti-IL-5 monospecific antibody
Irrelevant IgG2a antibody
Antibody concentration (μM)
G
Purified mouse B cells + IL-4
Purified mouse B cells
Purified mouse B cells + IL-4
+ irrelevant IgG2a antibody
Purified mouse B cells + IL-4
+ anti IL-4Rα monospecific antibody
Normalized To Mode
MHCII-APC


A
IL5
IL4Rα
VH
VL
CH1
CL
CH2
CH3
knobs into holes mutations
(a) Antibody mixture 1 (co-expression of the two mutated HCs and the two LCs of the anti-IL-4Rα and anti-IL-5 monospecific antibodies in one cell results in four combinations)
VHH anti-IL-4Rα monospecific antibody purification column
(b) Antibody mixture 2
VHH anti-IL-5 monospecific antibody purification column
(c) Endotoxin-free and pure anti-IL-4Rα/IL-5 bispecific antibody with a correct HC/LC chain pairing on each arm
B
Anti-IL-4Rα/IL-5 bispecific antibody
Anti-IL-4Rα monospecific antibody
Anti-IL-5 monospecific antibody
Counts
Deconvoluted mass (AMU)
C
1rst injection
2nd injection
Response units
Time (s)
1rst injection
2nd injection
Anti IL4Rα/IL5 bispecific antibody
IL5
Anti IL4Rα/IL5 bispecific antibody
IL4Rα Fc
Anti IL4Rα monospecific antibody
IL5
Anti IL5 monospecific antibody
IL5
D
1rst injection
2nd injection
Response units
Time (s)
1rst injection
2nd injection
Anti IL5 monospecific antibody
IL4Rα Fc
Anti IL4Rα/IL5 bispecific antibody
IL4Rα Fc
Anti IL4Rα/IL5 bispecific antibody
IL5
Anti IL4Rα monospecific antibody
IL5


A
Antibody treatment
(intraperitoneal)
d-1
d0
d1
d6
d7
d8
d9
d10
d14
Sensitization
(intratracheal;
1 µg HDM)
Challenge
(intranasal;
10 µg HDM)
Analysis
B
HDM/HDM/Irrelevant IgG2a antibody
HDM/HDM/Anti-IL-4Rα monospecific antibody
HDM/HDM/Anti-IL-5 monospecific antibody
HDM/HDM/Anti-IL-4Rα/IL-5 monospecific antibodies
Number of alive cells in BAL fluid
2.5×10^6
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0
ns
ns
ns
ns
ns
ns
***
*
**
****
****
****
Macrophages
Neutrophils
Lymphocytes
Eosinophils